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Review

The use of freshwater planarians in environmental toxicology studies: Advantages and potential

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ABSTRACT

Regarding the humane use of animals in scientific research, invertebrates are often recommended in toxicological studies. “Freshwater planarians” refers to numerous free-living freshwater members of the Class “Turbellaria” of the phylum Platyhelminthes. This group of invertebrates has received extensive attention from biologists for many years because of their unique biological characteristics, such as the primitive form of the central nervous system and notable capability to regenerate tissues. Using freshwater planarians as test animals in chemical toxicity studies has grown in popularity since the 1960s. Results from various toxicological experiments have collectively suggested that freshwater planarians can serve as not only alternative models for chemical toxicity screenings in laboratories but also as potential bioindicators for the quality of freshwater environments. However, thus far, no standardized battery of tests for conducting toxicological studies that includes freshwater planarians has been proposed. This paper comprehensively reviews the toxicological information obtained from chemically exposed planarians and proposes practical factors for consideration in toxicity experiments with freshwater planarians as test organisms.

1. Introduction

Following the guidelines on the humane use of animals in scientific research, also known as the three Rs (replace, reduce, and refine) (Russell and Burch, 1959), non-mammalian organisms are often recommended as surrogates for mammals in toxicological studies (Clay, 1996). Among such organisms, invertebrate species usually receive the most attention because they not only appear to be useful models for linking the sub-organismal effects of toxicants to changes at the population and community levels but also raise fewer societal concerns than do vertebrates (Lagadic and Caquet, 1998). The relatively high sensitivity of invertebrates to environmental chemicals makes them promising screening tools for predicting the acute toxicities of pollutants in relation to mammals (Calleja et al., 1994; Neuhauser et al., 1985, 1986). Moreover, because of their unique biological characteristics, such as relatively simple body structures and systems, shorter life cycles, and diverse reproductive strategies, invertebrates are useful experimental organisms to study biological and toxicological questions that are more difficult or time consuming to investigate by using mammalian systems. As the basic knowledge of the biology and physiology of invertebrate species is sufficient to assess the effects of a given chemical and to evaluate differences between invertebrates and vertebrates, replacing vertebrate animals with invertebrates in toxicity testing can likely yield more insightful results (Lagadic and Caquet, 1998).

Freshwater planarians are invertebrates that have received extensive attention from scientists over the preceding three decades (Fig. 1). Although planarian studies can be traced back to the early 1900s or before, the number of published works began to increase markedly in the 1950s. After 2000, the number of planarian-related publications increased substantially, with more than 50 papers having been published every year since. The number of planarian-related works is still increasing. In addition, since 2000, many review articles and books proposing the development of freshwater planarians as testing models in various scientific fields, such as aging (Oviedo et al., 2008a); pharmacology and drug abuse (Pagán, 2017; Pagan et al., 2009; Raffa and Rawls, 2009); human diseases (Lemieux and Warren, 2012; Prokai et al., 2013); ciliary assembly and motility (King and Patel-King, 2016); chemical toxicity, teratogenicity and tumorigenicity (Hagstrom et al., 2016a); neurotoxicology (Hagstrom et al., 2015, 2016a); carcinogenicity (Stevens et al., 2017) and stem cell biology and regenerative medicine (Gentile et al., 2011; Saló and Agata, 2012; Sheiman and Kreshchenko, 2015; Simanov et al., 2012), have been published. Such works indicate that the usefulness of freshwater

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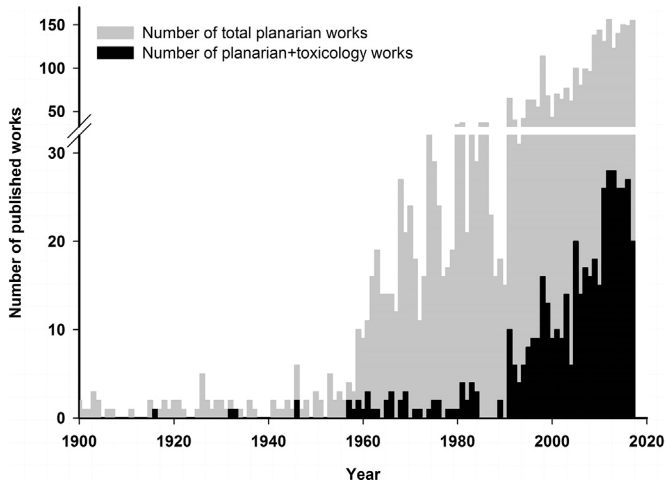

Fig. 1. Temporal changes in the number of journal articles reporting freshwater planarian biology (gray bars) and specific toxic effects on planarians (black bars) (1900–2017). The literature search was conducted using Web of Science with the following keywords: ((((planarian) OR flatworm) NOT marine) NOT parasitic) for the number of journal articles, and (((((planarian) OR flatworm) NOT marine) NOT parasitic) AND (toxic OR effect)) for the toxic effects.

planarians in biological research has been widely recognized by scientists in various fields.

Use of freshwater planarians as test animals in the field of toxicology started to become more common in the 1960s (Fig. 1). The literature search was conducted using Web of Science with the following keywords: (((((planarian) OR flatworm) NOT marine) NOT parasitic) AND (toxic OR effect)). The number of toxicological studies using freshwater planarians increased in the 1990s. Using freshwater planarians in toxicological studies appears to be promising. Freshwater planarians are often used to examine the toxicities of xenobiotic chemicals, especially those generated from human activities. Moreover, freshwater planarians have been suggested as invertebrate bioindicators for the quality of freshwater environments (Knakievicz, 2014).

Experimental results on the sensitivity and toxicological responses of freshwater planarians to environmental pollutants have increased over the preceding three decades. This paper comprehensively reviews these results. The first section of this review paper addresses the systematics and general biology of freshwater planarians to provide readers with background knowledge regarding these unique animals. In the second section, some critical advantages of using planarians in toxicological studies are addressed, and planarian-derived experimental systems are introduced. In the third section, studies are reviewed to provide information regarding planarians' sensitivity and toxicological responses to various classes of environmental pollutants. In the final section, recommendations for developing toxicological methods involving freshwater planarians for toxicological studies are discussed.

2. Systematics and biology of freshwater planarians

Freshwater planarian is the common name used for a wide range of freshwater and free-living members of the traditional taxon "Turbellaria" in Platyhelminthes, especially those placed in the order Tricladida (Ehlers, 1984; Noreña et al., 2015; Schockaert et al., 2008). The scheme of the current phylogenetic relationships of major taxa in Platyhelminthes, including turbellarians, is available from the turbellarian taxonomic database (http://turbellaria.umaine.edu) and some published works (Noreña et al., 2015; Schockaert et al., 2008). Thus far, at least 15 species of freshwater planarians have been used as test animals in toxicological studies, as listed in Table 1 alongside their current taxonomical status. Notably, the systematics of some species has been revised on the basis of new evidence; for example, *Girardia* and *Schmidtea* were considered subgenera of *Dugesia* until 1991, when they were elevated to independent genera (Vries and Sluys, 1991). To prevent misunderstandings regarding species identification in laboratory model systems, especially in comparative studies, authors and researchers prefer to use all available updated scientific names in their works to enable readers to understand linkages between old and new names.

Freshwater planarians exhibit worldwide distribution, are a part of the benthos, and inhabit floating vegetation in unpolluted freshwater environments (Noreña et al., 2015; Schockaert et al., 2008). Their role in the food web is that of a predator of other aquatic invertebrates in their surrounding habitat. The body structure of freshwater planarians is simple but has some unique biological characteristics. They are acoelomates; the free space unoccupied by organs (e.g., intestines and reproductive organs) is filled with the connective tissue or parenchyma. In addition to serving as a matrix to maintain organization, the parenchyma in planarians houses stem cells or neoblasts and is crucial for tissue regeneration (Sheiman and Kreshchenko, 2015). Freshwater planarians have an obvious bilobed neuronal cluster located in the head section. This neuronal cluster, or cephalic ganglion, fulfills the criteria for the definition of the brain in animals (Sarnat and Netsky, 2002). Thus, the central nervous system present in vertebrates may have evolved from its ancestor in planarians (Sarnat and Netsky, 2002); however, this idea was not supported by phylogenetic data (Northcutt, 2012). In addition, free-living freshwater planarians are hermaphrodites, and their reproduction can be either sexual or asexual. For a complete introduction of the bodily structure and physiology of turbellarians, see Noreña et al. (2015).

3. Using freshwater planarians in toxicological studies

3.1. Advantages of using freshwater planarians in toxicological studies

3.1.1. Being an representative invertebrate in aquatic environments

Many freshwater invertebrate models, such as annelids (Famme and Knudsen, 1985), arthropods (Gélinas et al., 2013), and bivalves (Faria et al., 2009), have been developed to assess the toxicity and ecological impact of environmental chemicals. In the food web, many of these aquatic invertebrates are decomposers or primary consumers that feed on organic debris or algae. By contrast, freshwater planarians are predators that assume the role of secondary consumers that mainly feed on other aquatic invertebrates. Collecting toxicity data from organisms located in multiple nodes of the food web is critical to ecotoxicologists and national environmental protection agencies for the comprehensive assessment of the impact and risk of environmental chemicals on the aquatic ecosystem.

3.1.2. Ease of acquisition

For research purposes, freshwater planarians can be easily acquired either commercially or collected from the wild. In the wild, they can be collected in bulk from the benthos (under rocks or fallen trunks) of unpolluted freshwater areas or by setting a trap with the fresh liver as bait (Noreña et al., 2015). Freshwater planarians are slow-moving animals with negligible resistance to salinity, rendering their long distance and transoceanic dispersal unlikely (Knakievicz, 2014). Therefore, each species of freshwater planarian generally has restricted geographical distribution. Predictably but notably, selections of planarian species by researchers for related studies have exhibited geographical dependence (Table 1). In other words, planarian researchers often use species that are native to areas in which they are located for easy acquisition.

3.1.3. Low maintenance cost

Freshwater planarians can be cultured easily (Noreña et al., 2015). Once in the laboratory, they can be stored in dechlorinated tap water, spring water, or reconstituted freshwater. Compared with established

Table 1
Systematics and geographical distributions of freshwater planarian species often used in toxicological studies by research teams from various regions.

Planarians		Location of research team(s)		Reference
Scientific name	Geographical distribution reported[a]	Country	Continent	
Dendrocoelidae				
Dendrocoelopsis vaginata	United States	United States	North America	Rivera and Perich (1994)
Planariidae				
Phagocata gracilis	United States	USA	North America	Onwumere and Wells (1983)
P. velata	United States	USA	North America	Phillips et al. (1974)
Polycelis felina	Algeria; Belgium; Germany; Italy; Macedonia; Morocco; Slovakia; Switzerland; United Kingdom	Croatia	Europe	Horvat et al. (2005)
P. tenuis	Finland; Macedonia; Netherlands; United Kingdom	Belgium	Europe	Indeherberg et al. (1999)
Dugesiidae				
Cura foremanii	Canada; France; United States	United States	North America	Rivera and Perich (1994)
Dugesia bengalensis	India	India	Asia	Mitra et al. (2003)
D. etrusca	Italy	Italy	Europe	Calevro et al. (1999)
D. gonocephala	Albania; Algeria; Angola; Belgium; Black Sea; China; Democratic Republic of the Congo; France; Hungary; Italy; Kenya; Macedonia; Morocco; North Korea; Poland; Slovakia; Switzerland; Taiwan; Tunisia	Italy	Europe	Venturini et al. (1989)
D. japonica	China; Japan; Savu Sea; South Korea; Taiwan	China	Asia	Zhang et al. (2014)
		Taiwan	Asia	Wu and Li (2017)
		Japan	Asia	Nishimura et al. (2011)
		Mexico	North America	Garcia-Medina et al. (2013)
D. subtentaculata	Algeria; Bulgaria; France; Spain	Portugal	Europe	Rodrigues et al. (2016)
Girardia dorotocephala	Canada; Mexico; United States	USA	North America	Hall et al. (1986a, 1986b)
G. schubarti	Brazil	Brazil	South America	Guecheva et al. (2001)
G. tigrina	Black Sea; Brazil; Canada; France; Gemany; Iraq; Ireland; Italy; Japan; Mexico; Netherlands; United States; Uruguay	Brazil	South America	Knakievicz and Ferreira (2008)
		Russia	Asia	Kustov et al. (2014)
		Netherlands	Europe	Indeherberg et al. (1999)
Schmidtea mediterranea	France; Italy; Spain	Belgium	Europe	Plusquin et al. (2012)
		Portugal	Europe	Ofoegbu et al. (2016)

[a] Based on the World Register of Marine Species on-line database (WoRMS; http://www.marinespecies.org/index.php) (accessed in March 2018).

Table 2
Comparison of maintenance requirements for culturing freshwater animals in laboratories.

Animal	Japanese Medaka[a]	Zebrafish[b]	Daphnids[c]	Planarians[d]
Rearing system	Tanks equipped with filtration units or recirculation systems	Tanks equipped with filtration units or recirculation systems	Containers with still water	Containers with still water
Housing density (individual/liter)	Larvae: 5; Juvenile: 1.4–2.1; Spawning: 0.3	5	12.5	200–350
Water Source	Spring water, well water, controlled surface water, dechlorinated tap water, and etc.	Spring water, well water, controlled surface water, dechlorinated tap water, and etc.	Reconstituted water	Spring water, well water, controlled surface water, dechlorinated tap water, reconstituted water
Environmental condition	Temp.: 22–28 °C; Photoperiod: 16 h(L)/8 h(D) Hardness: 40–300 mg $CaCO_3$/L;	Temp.: 28.5 °C; pH: 7.0–8.0; DO: 6.0 mg L^{-1}.	Temp.: 20 ± 1 °C; Photoperiod: 16 h(L)/8 h (D).	Temp.:17–20 °C; Photoperiod: 12 h(L)/12 h(D); pH: 7.5, approximately.
Food source	Live or frozen food(*e.g. Artemia, Tubifex, and Daphnia*), commercial fish food, and etc.	Live or frozen food(*e.g. Artemia, Tubifex, and Daphnia*), commercial fish food, and etc.	Algae (*Chlorella vulgaris*), Baker's yeast	Raw chicken or beef livers, boiled egg yolk
Feeding frequency	twice each week day; once a day on weekends	twice or three times a day	Once daily	once or twice a week

[a] Danny et al. (1991).
[b] Reed and Jennings (2011).
[c] Heckmann and Connon (2007).
[d] Oviedo et al. (2008b).

aquatic animal models (e.g., Japanese medaka, zebrafish, and daphnid), maintaining colonies of freshwater planarians in laboratories is cost effective because they can be stored in a low volume of culture media with high density (Table 2). In general, the difficulty and cost of maintaining freshwater planarians in a laboratory are low. The inexpensive raw liver and egg yolk are the most common major food sources for planarians stored in laboratories. Water change is required after every feeding. Freshwater planarians can tolerate a wide range of temperatures but are commonly stored at room temperature. Because of their small size, only a low volume of test medium and a small volume of test chemicals are required, thereby reducing not only the cost of toxicological testing but also the volume of waste water discharged during experimentation.

3.1.4. Availability of physiological and genetic information

Freshwater planarians are attractive test animals because they have not only some unique biological characteristics (e.g., tissue regeneration capability) but also some physiological systems that are similar to those in mammals (e.g., the neural system). Knowledge regarding the bodily structure and physiology of freshwater planarians has also been established (Noreña et al., 2015). In addition, databases providing genomic information on some species, such as *Schmidtea mediterranea* (http://smedgd.stowers.org) (Robb et al., 2015) and *Dugesia japonica* (http://planarian.bio.keio.ac.jp/index.html), have been released. Thus, many adverse effects observed in xenobiotic-exposed planarians can be extensively investigated using physiological or molecular biological scales to provide more mechanistic insights into the toxicity of chemicals.

3.2. Experimental systems derived from freshwater planarians

Freshwater planarians can be used in the development of experimental systems for toxicological research. Obtaining experimental systems with planarians is easier and more convenient than obtaining them from vertebrate models; this is one advantage of using freshwater planarians in scientific research.

3.2.1. Whole-animal in vivo system

The most commonly used freshwater planarians are live intact individuals, or in vivo systems. They are often used to study the toxic effects of waterborne chemicals that are moderately or highly water soluble, including environmental pollutants, addictive drugs, and pharmaceuticals (Alonso and Camargo, 2011, 2015; Buttarelli et al., 2000; Grebe and Schaeffer, 1991a; Guecheva et al., 2003; Horvat et al., 2005; Knakievicz and Ferreira, 2008; Kovačević et al., 2009; Li, 2013a; Pagan et al., 2015; Rawls et al., 2011; Rodrigues et al., 2016; Schaeffer, 1993; Wu et al., 2012a, 2014, 2015, 2012b; Wu and Li, 2017; Zhang et al., 2016, 2014). Live intact planarians are usually exposed to chemicals through direct soaking in the test solution followed by sampling and toxicity assessment. Generally, fluids penetrate into freshwater planarians' bodies through cutaneous diffusion through the body wall (Kapu and Schaeffer, 1991).

Although planarians can be exposed to chemicals through ingestion, this method is not commonly applied. Planarians are exposed to xenobiotic chemicals by being fed food (e.g., a piece of the beef liver) perfused in advance with the target chemical (Phillips et al., 1974). Exposing freshwater planarians to xenobiotics through ingestion can be conducted only on worms with normal feeding behaviors and a functional mouth (and pharynx). The homogeneous degree of the target chemical within the carrier food and the feeding efficiency of planarians can influence the amount of the given chemical that the planarians actually ingest, leading to mis-estimation of the exposure dose.

3.2.2. In vitro systems

3.2.2.1. Neoblasts. Compared with whole-animal in vivo experiments, in vitro studies generally provide more mechanistic insights into toxic effects caused by xenobiotics. The first attempt at the in vitro cultivation of planarian cells or tissues was undertaken by Murray (1927); no effort has since been made to establish a permanent planarian cell line or primary cell culture (Schürmann and Peter, 2001) except for neoblasts, which play a crucial role in planarian tissue regeneration (Behensky et al., 2001; Hori and Kishida, 1998; Lopes et al., 2015; Schürmann and Peter, 2001; Sengel, 1960).

Planarian neoblasts appear as small (i.e., 5- to 10-µm in diameter) and roundish cells with a thin cytosol rim, many free ribosomes, few discernible organelles, prominent chromatoid bodies, and a large nucleus with little heterochromatin (Reddien and Alvarado, 2004; Rink, 2013). They are considered useful materials to investigate the potential genotoxicity, teratogenicity, tumorigenicity, and developmental toxicity of environmental chemicals (Best and Morita, 1982). For toxicological studies, planarian neoblasts can be obtained from exposed planarians (Knakievicz et al., 2008; Knakievicz and Ferreira, 2008) or regeneration buds in treated planarian fragments in which the regeneration process has occurred (Best and Morita, 1982; Calevro et al., 1998; Kalafatić et al., 2004).

3.2.2.2. Amputated fragments. Tissue regeneration in amputated planarian fragments, particularly head formation from a tail fragment, is a useful indicator to evaluate the toxicity of environmental chemicals (Best and Morita, 1982; Calevro et al., 1998; Li, 2014). According to the external appearance of head regeneration in decapitated planarians, the formation process can generally be divided into several stages: (I) the presence of a wound or lesion, (II) the appearance of a blastema, (III) blastemal assuming its normal shape, (IV) the presence of an eye spot, (V) the presence of an auricle, and (VI) the complete formation of a head (Calevro et al., 1998; Li, 2014). Through daily observations of the progress of head formation and by scoring each regeneration stage in a decapitated planarian exposed to the test chemical in question, activation or inhibitory effects of tissue regeneration can be quantified. Abnormalities of regenerated tissues, if any, can serve as useful indicators of the toxicity of the test chemical. In addition, after surgery, all amputated planarian fragments remain alive; thus, a comparison between the toxic responses of different fragments treated with the same toxicant is possible (Wu et al., 2011).

3.2.2.3. Tissue homogenates. Planarian tissue homogenates can be used to examine the direct effects of xenobiotics on the biochemical functions of cellular components, such as the activities of cytosolic or membrane-bound enzymes. After a tissue homogenate has been obtained, exposure can be conducted through direct incubation in a solution containing the studied xenobiotic chemical followed by determination of the function of the cellular component of focus (Hagstrom et al., 2016b; Wu et al., 2011; Wu and Li, 2015).

4. Are freshwater planarians useful bioindicators for environmental chemicals?

4.1. Sensitivity of freshwater planarians to environmental chemicals

4.1.1. Acute toxicity results

The acute toxicities of various metals and pesticides in relation to freshwater planarians were summarized by Knakievicz (2014); the present review is further updated from the literature shown in Supplementary Table S1. Of the metals tested, freshwater planarians are more susceptible to cadmium (Cd), copper (Cu), mercury (Hg), and tributyltin than to other metals, with median lethal concentration (LC_{50}) values at ppb or sub-ppm levels. Regarding pesticides, freshwater planarians are generally more susceptible to most insecticides than to fungicides and herbicides; the LC_{50} values of freshwater planarians after short-term exposure are at ppb or sub-ppm levels for insecticides and at ppm levels for fungicides and herbicides.

Ammonia and nitrite generally result from several anthropogenic sources in freshwater ecosystems, and their toxicities in relation to freshwater planarians have been estimated (Alonso and Camargo, 2008, 2006, 2015). Un-ionized ammonia at concentrations of higher than $0.3\,mg\,L^{-1}$ caused a significant increase in the mortality of *Polycelis felina* after 21 days of treatment (Alonso and Camargo, 2015). The 24-, 48-, 72-, and 96-h LC_{50} values of nitrite in relation to *P. felina* were 787, 141, 79.8, and $60.0\,mg\,L^{-1}$, respectively (Alonso and Camargo, 2006).

Piontek (1999) conducted a comparative study to investigate the acute toxicities of 16 organic substances in relation to intact and cut samples of *Girardia tigrina*. Aromatic compounds were estimated to be highly toxic to this species. In addition, the acute lethality of several emerging contaminants detected in aquatic environments has been investigated in freshwater planarians (Li, 2012a, 2013a, 2013b, 2014, 2008, 2012b) (Supplementary Table S2). Freshwater planarians exhibit considerable divergence regarding their susceptibility to emerging contaminants, with LC_{50} values within the range of hundreds of ppb to hundreds of ppm.

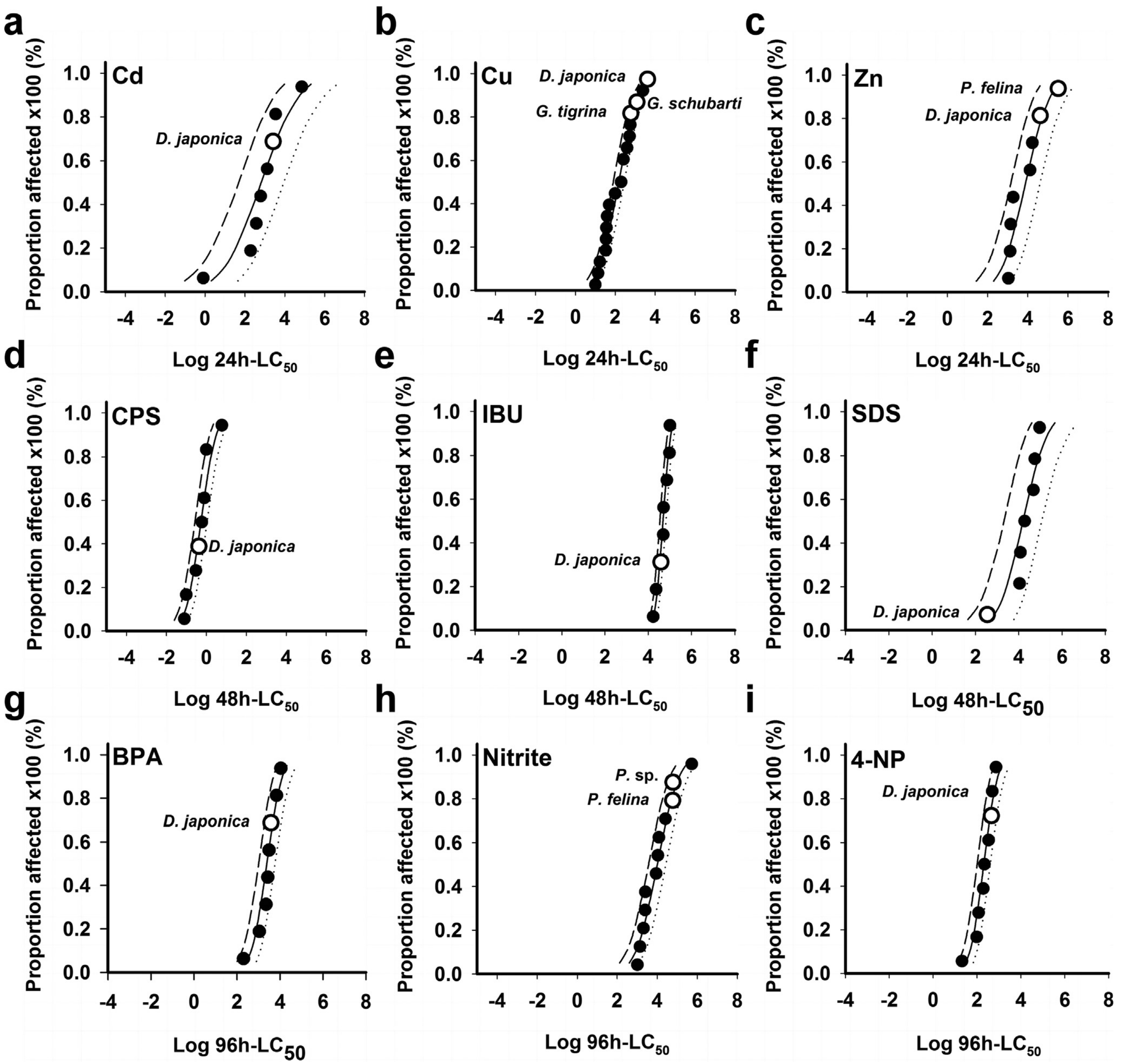

Fig. 2. Species sensitivity distributions (SSDs) of median lethal concentration (LC_{50}) values for freshwater invertebrates exposed to (a) cadmium (Cd), (b) copper (Cu), (c) zinc (Zn), (d) chlorpyrifos (CPS), (e) ibuprofen (IBU), (f) sodium dodecyl sulfate (SDS), (g) bisphenol A (BPA), (h) nitrite, and (i) 4-nonylphenol (4-NP). ○, data from freshwater planarian species; •, data from other freshwater invertebrates.

4.1.2. Comparisons of the sensitivity of freshwater planarians vs. other freshwater animals

To compare the sensitivity to environmental chemicals between planarians and other freshwater invertebrates, we collected LC_{50} values of environmental chemicals from the literature. Datasets with sufficient numbers of tested species (≥ 8), including those of conventional and emerging contaminants, were used to generate species sensitivity distribution (SSD) models through a program downloaded from the United States Environmental Protection Agency website (https://www.epa.gov/exposure-assessment-models/species-sensitivity-distributions). The resultant models indicated that compared with other freshwater invertebrates, most of the tested freshwater planarians are more or less sensitive to many pollutants (Fig. 2; Supplementary Table S3); moreover, some species of planarians (e.g., *D. etrusca*) are more susceptible than their relatives (Supplementary Table S1).

Daphnid species are common invertebrate models in environmental and ecotoxicological studies. The sensitivities of freshwater planarians and daphnids to metals, pesticides, organic chemicals, and other contaminants vary depending on the species and chemicals examined; however, range of such variance is small (Alberdi et al., 1996; Li, 2013a; Piontek, 1999; Servizi et al., 1987; Wu and Li, 2017). A comparative study observed that the tolerance of planarians to nitrite exposure was higher than that of amphipods, crayfish, and mayflies but lower than that of snails (Alonso and Camargo, 2006). These findings suggested that the sensitivities of planarians to many environmental chemicals fall within the sensitivity ranges of other freshwater invertebrates and are similar to those of the most commonly-used invertebrate models.

4.2. Toxicological responses of freshwater planarians after exposure to environmental chemicals

In addition to acute lethality, many studies have investigated the sublethal or chronic effects of environmental chemicals on freshwater planarians. In such studies, planarians are often exposed to chemicals at lower doses, environmentally-relevant or not. Some selected responses at various biological levels are commonly monitored in exposed planarians. Subsequently, responses that exhibit strong dose and time-dependency are often proposed as potential biomarkers for the existence of toxic substances in the environment. From a toxicological perspective, planarian responses after exposure to chemicals may provide critical implications for understanding mechanisms underlying toxicity.

4.2.1. Teratogenicity and developmental toxicity

The processes of tissue regeneration and remodeling in planarians are similar to those of embryogenesis in higher animals. Since induced neoplasms, malformations, and lethal growths were observed in freshwater planarians exposed to known carcinogens or teratogens nearly half a century ago (Foster, 1969, 1963), they have been considered useful models for assaying the teratogenicity and developmental toxicity of chemicals (Best and Morita, 1982, 1991; Schaeffer, 1993).

Head regeneration in decapitated planarians is the primary endpoint when using planarians for teratogenicity testing (Collins, 1987; Goss and Sabourin, 1985). Both quantitative (e.g., the time taken to obtain identified developmental markers) and descriptive (e.g., types of morphological abnormalities) results of cephalic regeneration have proven informative and have been used extensively as teratogenicity endpoints (Best and Morita, 1982; Calevro et al., 1998; Hagstrom et al., 2015; Li, 2014; Sabourin et al., 1985). Delayed or inhibited head regeneration occurs in many planarian species, if not all, during dose-dependent exposure to various chemicals (Calevro et al., 1998; Knakievicz and Ferreira, 2008; Li, 2014; Medvedev and Komov, 2005; Ofoegbu et al., 2016; Schürmann and Peter, 2001; Van Huizen et al., 2017; Zhang et al., 2013). The mitotically-active neoblast should be involved in the tissue regeneration process in planarians; however, the relationship between neoblast activity and regeneration outcome remains unclear because opposite responses of its division have been observed in different species of planarians treated with the same toxicant (Kalafatić et al., 2004; Plusquin et al., 2012). In amputated *D. japonica* treated with the pesticide permethrin, delayed eye regeneration was observed without a significant change in the blastema growth rate compared with that in untreated worms (Hagstrom et al., 2015).

Some teratogenic responses, such as lesions, resorption, head dissolution, and supernumerary eyes and heads, were observed in intact planarians exposed to teratogens (Best and Morita, 1982). Moreover, Hagstrom et al. (2015) observed that some endpoints, such as brain morphology, were less affected in intact planarians than in regenerating worms treated with the same chemical at the same dose.

4.2.2. Tumorigenicity and carcinogenicity

For planarians, chemical tumorigenicity is often used to describe the capability of chemicals to result in abnormal growth of body parts or tumor-like growths (neoplasia) (Schaeffer, 1993). Histopathologically, at least two types of tumors can be characterized in known carcinogen-treated planarians: (1) the benign but persistent “Type I” tumor and (2) the invasive and malignant “Type II” tumor (cancer) (Hall et al., 1986a, 1986b). Various locations where tumor-like growths occurred in planarians after various exposures have been observed and described by some toxicologists (Hall et al., 1986a, 1986b; Tehseen et al., 1992). After reviewing the tumorigenicity of known mammalian carcinogens, non-carcinogens, and radiation in planarians, Schaeffer (1993) concluded that regardless of the type of tumor and the location in which it occurred, freshwater planarians exhibit high specificity and sensitivity for tumorigen screening, including those in environmental field samples. Voura et al. (2017) found that neoblast activity responded to Cd-induced tumor growth and metalloproteinases were required for the progression of cancer in exposed planarians. Furthermore, in one study, differences in the proliferation patterns of neoblasts and phenotypes appeared in regenerating *Schmidtea mediterranea* exposed to genotoxic carcinogens, nongenotoxic carcinogens, and non-carcinogens; Stevens et al. (2017) proposed a workflow to detect and classify carcinogenic chemicals.

4.2.3. Genotoxicity

Exploration of the genotoxic effects of chemicals on planarians has increased since the early 2000s. Chemical genotoxicity is usually examined in exposed intact planarians through the single-cell gel test or comet assay. Until now, DNA damage has been demonstrated in planarians exposed to some metals and pesticides (García-Medina et al., 2013; Guecheva et al., 2001; Horvat et al., 2005; Ofoegbu et al., 2016). By amplifying random DNA fragments with single short primers of arbitrary nucleotide sequences under low annealing conditions, the randomly-amplified polymorphic DNA method has the potential to detect a broad scope of DNA damage and mutation. Recently, Zhang et al. (2017) successfully used this method to evaluate the genotoxic potential of an urban river on freshwater planarians. The researchers suggested that this method may be applicable for testing potential chemical genotoxicity and monitoring environmental quality.

In *G. tigrina* and *G. schubarti* treated with known carcinogens, including γ-rays, methyl methanesulphonate (MMS), and cyclophosphamide (CPA), aberrations in the chromosomes of these planarians were observed (Lau et al., 2007). *G. tigrina* generally exhibited higher sensitivity to the genotoxicity of tested agents than did *G. schubarti*. Additionally, elevations in the micronucleus (MN) frequency—another reflection of chromosomal aberrations during mitosis—were reported in the neoblasts of *G. schubarti* and *G. tigrina* after treatment with γ-rays, MMS, and CPA at non-cytotoxic levels (Knakievicz et al., 2008)

4.2.4. Neurotoxicity and effects on locomotion and neurobehaviors

Freshwater planarians have well-developed neural systems similar to those of mammals (Buttarelli et al., 2008; Noreña et al., 2015). Because of the capability of freshwater planarians to regenerate tissue after surgery, they provide toxicologists an opportunity to study the developmental neurotoxicity of toxic chemicals. Papenfus (1983) treated decapitated asexual *D.* (*G.*) *dorotocephala* with methylmercury (MMC) for 2 weeks and found that their righting time increased and their motility and predatory activity decreased when their brains were developing. A further histopathological examination conducted through electron microscopy revealed declined synaptic density in the regenerated heads of MMC-treated planarians. Thus, the abnormal behaviors observed in planarians treated with MMC may have resulted from the effects of MMC on synapse development (Best and Morita, 1991). The brain plays a functional role in activating planarian motility (Kato et al., 2004). The locomotion and behavior of decapitated planarians are regulated by the reconstructed dopaminergic neural network of the regenerated brain (Nishimura et al., 2007). Thus, any chemical-mediated disturbance in brain and neural system regeneration in freshwater planarians can be assayed by measuring their motility.

Planarian motility, also referred to as “mobility,” “behaviors,” and “locomotion activity,” can be quantified by measuring the total distance that a planarian moves over a known observation period; this is sometimes referred to as the planarian locomotor velocity (pLMV) method (Pagán et al., 2006; Raffa et al., 2001). Altered motility has been observed in intact planarians exposed to various environmental chemicals (Knakievicz and Ferreira, 2008; Li, 2012b; Ofoegbu et al., 2016; Plusquin et al., 2012; Rodrigues et al., 2016; Wu et al., 2014; Zhang et al., 2013); however, the motility of intact planarians is still considered the integration of their biochemical and physiological processes (Rodrigues et al., 2016). In contrast to cases of decapitated planarians, the measured motility of exposed intact planarians is difficult to mechanistically or specifically link to the effect on their neural systems. Instead of using the traditional pLMV method, which relies on manual observation, a recent study applied a real-time center of mass tracking system to determine the motility, locomotion type, and thermotaxis of exposed planarians (Hagstrom et al., 2015); this has rendered the development of planarians as a model system for neurotoxicology and enabled the medium-throughput screening of potentially-toxic substances in a time- and cost-efficient manner (Hagstrom et al., 2016a).

Some morphological characteristics and neurobehaviors of intact planarians exposed to environmental chemicals can be linked to disturbances in their neural systems; this finding was initially derived from some pharmacological studies that have used freshwater planarians as in vivo models. Most neurotransmitters and their receptors found in mammals have also been characterized in planarians (Buttarelli et al., 2008). Several distinct behavioral patterns have been characterized in planarians exposed to pharmaceuticals acting on neural transmissions (Buttarelli et al., 2000; Carolei et al., 1975; Farrell et al., 2008; Palladini et al., 1996; Venturini et al., 1989). Distinct behavioral patterns in exposed planarians were first characterized in the early 1990s (Grebe and Schaeffer, 1991a, 1991b). Behavioral responses of planarians after exposure to toxicants were grouped into five categories, specifically locomotive, morphological, neurological, morbidity, and protective responses. Similarly, intense twisting, incoherent reactions to mechanical stimuli, contractions, mucus secretion, and deformations were documented in aluminum (Al)-treated *P. felina* (Kovačević et al., 2009). More recently, Wu et al. (2014) treated *D. japonica* with high levels of Cd and observed not only distinct neuro-behaviors but also altered activity of enzymes involving the metabolisms of neurotransmitters. Wu et al. (2015) further demonstrated disturbances in neurotransmitter levels and their metabolic enzyme activity in *D. japonica* exposed to Cd at sublethal concentrations.

4.2.5. Reproductive toxicity

The reproduction of aquatic organisms can be a sensitive endpoint to evaluate the effect of environmental chemicals on populations of aquatic organisms. Because freshwater planarians can reproduce sexually (Hoshi et al., 2003; Kobayashi et al., 2002; Noreña et al., 2015), they may constitute a useful model for testing the reproductive toxicity of environmental pollutants. Reductions in fecundity (the number of cocoons produced) and fertility (the number of hatchlings produced) have been demonstrated in metal-exposed planarians (Indeherberg et al., 1999; Knakievicz and Ferreira, 2008). The mechanism underlying decreased sexual reproduction remains unclear. It is likely a result of the indirect effects on neurotransmitters and neurohormones involved in the proliferation of neoblasts and germinative cell precursors (Knakievicz, 2014) or disruption of energy reserves (Vu et al., 2017). Long-term exposure to environmental chemicals, such as endocrine-disrupting chemicals (EDCs), can also disturb hormone regulation in aquatic animals. However, the hormone regulation for sexual development in freshwater planarians is still unclear. There are still limitations on using planarians to assess the effects of EDCs or be good indicators of EDC effects for other phyla.

4.2.6. Biochemical responses

An imbalanced oxidative status can be observed in many stressed organisms, including freshwater planarians. Alterations in the activities of antioxidant enzymes, changes in the glutathione content, and the accumulation of malondialdehyde, a biomarker for lipid peroxidation, have been demonstrated in several species of freshwater planarians exposed to chemicals that can induce oxidative stress (García-Medina et al., 2013; Guecheva et al., 2003; Li, 2008; Wu et al., 2012a; Zhang et al., 2016, 2014). Plusquin et al. (2012) determined the enzyme activities and transcript levels of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in Cd-treated *S. mediterranea*. Changes in GPx and SOD activities were observed during the exposure period; however, a considerable alteration in gene expression was only observed in GPx, indicating that the activities of some antioxidation enzymes were modified post-transcriptionally in planarians.

Widely-defined metallothioneins (MTs) are metal-thiolate polypeptides that resemble equine renal MTs and are present in various organisms. They are inducible and play crucial roles in the regulation of essential metals and detoxification of the unusual entrance of essential and non-essential metals (Roesijadi, 1992). The relationship between tissue Cd concentrations and induced MT levels was demonstrated in Cd-treated *D. japonica* (Wu et al., 2012a). Notably, MT levels and the induction of MT by Cd exhibited different patterns in head and tail fragments; this was considered a possible cause of the fragment-specific distribution of Cd in planarian bodies (Wu et al., 2011). By contrast, in planarians treated with Cu, the tissue distribution of Cu and the patterns of MT induction did not differ between the head and tail (Wu et al., 2011). These findings suggest that at least in *D. japonica*, different metals are distributed with different patterns in various body portions.

Other biochemical markers that have been assayed in toxicant-exposed planarians include (but are not limited to) the expression of heat shock proteins (HSPs), activities of detoxification enzymes, and patterns of macromolecules. HSPs are protein families classified into four major groups based on their molecular weight, including hsp90, hsp70, hsp60, and low-molecular-weight stress proteins (Morimoto et al., 1990). Although HSPs constitutively expressed, their inductions are detectable in cells exposed to harmful stimuli; thus, they are often used as biomarkers for stressors (Welch, 1993). Inductions of HSP levels or gene expressions were observed in several species of planarians exposed to metals (Guecheva et al., 2003; Plusquin et al., 2012; Zhang et al., 2016); however, initial inhibitions in the transcript levels of HSP60 and HSP70 were also observed in Cd-treated *S. mediterranea* (Plusquin et al., 2012). Li (2016) determined the activities of phase I and II detoxification enzymes in planarians by directly measuring the disappearance of the enzyme substrate or the production of enzyme metabolites in planarian culture media. Changes in the activities of these enzymes were demonstrated after the planarians had been exposed to their known inducers or inhibitors. However, further application of this *in vivo* assay to test the impacts of other environmental chemicals on planarian detoxification enzymes is warranted. Finally, Mitra et al. (2003) observed that Cd enhanced bodily protein and lipid levels in treated *D. bengalensis*, followed by a gradual decline; the authors considered that this may have been a result of the disturbance of Cd in the uptake and metabolism of these macromolecules.

4.3. Comparisons among freshwater planarians and other aquatic invertebrate models

Due to advantages such as broad distribution, low difficulty in terms of maintenance and handling, small size, short life span, low genetic variability in some species, and ecological specificity, numerous freshwater invertebrates belonging to various taxonomic groups have been used as test animals for hazard identification, toxicity testing, and risk assessments of environmental chemicals (Lagadic and Caquet, 1998). However, invertebrates differ in some, if not all, biological characteristics such as organ and system constitution, physiological functions, and survival and reproduction strategies. The type of chemical toxicity that can be explored by invertebrates usually depends on the biological characteristics of the test animals used.

The types of chemical toxicity that have been explored using various freshwater invertebrates are summarized in Table 3. Although hydrozoans have been used to test teratogenicity, genotoxicity, behavioral toxicity, reproductive toxicity, and biochemical disturbance caused by chemicals (Bowden et al., 1995; Devaux and Larno, 1999; Fukuhori et al., 2005; Quinn et al., 2009; Zeeshan et al., 2016), they have not been used as test organisms for chemical tumorigenicity or neurotoxicity. Similarly, although many studies on various types of chemical toxicity have used freshwater annelids (Famme and Knudsen, 1985; Gerhardt, 2009; Jha et al., 1996; Rogge and Drewes, 1993; Ventura-

Table 3
Toxicities of environmental chemicals that have been investigated using aquatic invertebrate models.

Type of toxicity	Cnidarians	Planarians	Annelids	Bivalves	Crustaceans
Teratogenicity	V	V			V
Tumorigenicity		V		V	
Genotoxicity	V	V	V	V	V
Neurotoxicity		V	V	V	V
Behavioral toxicity	V	V	V	V	V
Reproductive toxicity	V	V	V	V	V
Biochemical disturbance	V	V	V	V	V

Lima et al., 2007), bivalves (Conners and Black, 2004; Faria et al., 2009; Gardner et al., 1991; Matozzo et al., 2005; Thain, 1986), and crustaceans (De Schamphelaere et al., 2004; Gélinas et al., 2013; Gómez-Oliván et al., 2014; Gauthier et al., 2016; Kikuchi et al., 2000; Lacaze et al., 2010; Mann et al., 2010; Oliveira et al., 2015; Reutgard et al., 2014; Ton et al., 2012), these organisms have not been used to examine the teratogenicity or tumorigenicity of chemicals. Freshwater planarians are notably the only group of freshwater invertebrates that have been used to study all types of toxicities listed in this paper; this implies that, compared with other freshwater invertebrates, freshwater planarians are more powerful test organisms for comprehensively evaluating the toxicity of environmental pollutants at various biological levels.

5. Considerations for using freshwater planarians in toxicity testing

Hagstrom et al. (2016a) highlighted the following three challenges that should be considered when using planarians in toxicological studies and hazardous chemical screening: (1) the lack of fully automated assays; (2) the need for a standardized battery of tests and one or two species for conducting toxicological studies; and (3) the need for further investigation to understand differences in toxicant targets and metabolism between planarians and mammals. Because some semi-automated assays that use planarians for toxicant screening are under investigation (Hagstrom et al., 2015), further development of a fully automated assay is expected. In addition, information on the differences in physiological functions, toxicant effects, and metabolism between planarians and more complex animals is currently being generated (Wu et al., 2015, 2012b). Although the need for a standardized battery of tests for conducting toxicological studies using planarians has recently been realized (Hagstrom et al., 2016a; Knakievicz, 2014), no such battery has yet been proposed. The following subsection provides suggestions regarding the development of a toxicity test for when freshwater planarians are used to investigate the toxicity of environmental chemicals.

5.1. Species

5.1.1. Differences in sensitivity among species

Because freshwater planarians represent a group of free-living turbellarians consisting of more than 400 known species (Schockaert et al., 2008), their diverse range of susceptibility to the same chemicals is not surprising (Supplementary Table S1). Regardless of experimental conditions used by different laboratories, some species are considerably more sensitive than are others. Under the same experimental conditions, Van Huizen et al. (2017) examined the acute toxicity of a fungicide, namely methylisothiazolinone, to five species of freshwater planarians and observed species-specific differences in terms of the planarians' sensitivity to the same toxicant.

5.1.2. Difference in toxicity among species

Table 4 summarizes the types of toxicity that have been evaluated in relation to various planarian species in the literature. Lethality, developmental toxicity, genotoxicity, neurotoxicity and behavioral changes, and biochemical responses caused by chemical exposures can be examined using most of these species, despite differences in sensitivity.

Chemical-induced tumor-like growths have been observed only in carcinogen-exposed *G. dorotocephala* (Hall et al., 1986a; Schaeffer, 1993) and *G. tigrina* (Voura et al., 2017). Altered neoblast activity has been observed in *G. tigrina* and *S. mediterranea* following carcinogen exposure; however, the accompanying growth of tumor-like tissues has appeared only in *G. tigrina* (Stevens et al., 2017; Voura et al., 2017). Chemical-induced tumorigenicity may be a species- or genus-specific response that occurs only in a limited number of species with the cause still unclear. Thus, until further investigation has been conducted, only *G. dorotocephala* and *G. tigrina* can be used as trusted models for studying chemical-induced tumorigenicity.

The effect of toxicity on sexual reproduction in planarians has been tested only on *G. tigrina* and *P. tenuis* (Indeherberg et al., 1999; Knakievicz and Ferreira, 2008); no relevant information regarding other species of freshwater planarians is available. However, the reproductive toxicity of chemicals in relation to planarians can be assayed in many species that produce cocoons and sexually-reproducing individuals, so long as sufficient numbers of such individuals can be obtained for testing.

5.2. Populations

Freshwater planarians are small and slow-moving animals, rendering their dispersal over long distances unlikely; however, the isolation of different populations is possible. Indeherberg et al. (1999)

Table 4
Chemical toxicities demonstrated in various species of planarians after treatment.

Planarians	Chemical toxicity						
	Lethality	Developmental toxicity	Tumor-like growth	Genotoxicity	Neurotoxicity and/or behavioral change	Reproductive toxicity	Biochemical responses
D. bengalensis							V
D. etrusca	V	V					
D. japonica	V	V		V	V		V
D. subtentaculata					V		
G. dorotocephala	V	V	V	V	V		V
G. schubarti	V			V			V
G. tigrina	V	V	V	V	V	V	
P. felina	V	V		V	V		
P. tenuis	V	V				V	
Ph. gracilis	V						
S.mediterranea	V	V		V	V		V

collected *P. tenuis* from five locations along the same river, including four sites that receive metal inputs and one in an unexposed tributary, and exposed the samples to Cd in the laboratory before quantifying their toxic responses. The researchers found that some toxicological responses of Cd-treated *P. tenuis* were distinguishable among different populations. Sensitivity to chemicals in a single planarian species may differ among isolated populations, possibly because of long-term adaptation to the environmental conditions of their habitats or pollution selection pressure (Indeherberg et al., 1999; Knakievicz, 2014).

5.3. Age

Because aging of asexual planarians is impossible, the influence of age on the results of toxicity tests remains unclear. By contrast, sexually-reproducing planarians of different ages exhibit varying sensitivities to chemicals (Supplementary Table S1). Compared with adults, the locomotion of newborns is more easily impaired by Cu (Knakievicz and Ferreira, 2008), thereby indicating that physiological differences may exist between younger and older planarians. At least, the micronucleus (MN) frequency in unexposed newborn planarians was higher than in adults (Knakievicz and Ferreira, 2008). Naturally, using a batch of planarians comprising individuals at different developmental stages to evaluate toxicity could yield inconsistent results, thereby causing over- or under-estimation of toxicity.

5.4. Sexual vs. asexual reproduction

Information regarding differences in sensitivity and toxicological responses to the same chemical between sexual and asexual planarians of the same species remains limited. Notably, the organ constitutions and gene expression patterns in sexual and asexual planarians of the same species are distinguishable from each other (Hase et al., 2004; Kobayashi and Hoshi, 2002; Noreña et al., 2015). Toxicological responses and sensitivity to the same chemical may not be comparable between sexual and asexual individuals of the same planarian species; however, further investigation is necessary to support this hypothesis.

5.5. Experimental systems

As stated in Sections 3.2 and 4.2, various experimental systems using planarians provide opportunities to evaluate different types of toxicity after the corresponding endpoints have been assayed. Notably, differences in sensitivity to the same chemical between intact planarians and their amputated fragments have been observed; however, the degrees of difference might have depended on the chemicals tested (Knakievicz and Ferreira, 2008; Wu et al., 2011).

5.6. Solvents used in the preparation of test chemicals

Water (natural or reconstituted), ethanol, and dimethyl sulfoxide (DMSO) are some frequently-used solvents used to dissolve chemicals for conducting toxicity tests with aquatic organisms. However, ethanol and DMSO can exert adverse effects on freshwater planarians.

Exposure to ethanol with a concentration higher than 3% for 24 h is lethal for *S. mediterranea* (Lowe et al., 2015). In the same study, in amputated *S. mediterranea*, exposure to 1% ethanol induced a delay in the reacquisition of behavior during head regeneration. Tallarida et al. (2014) exposed *G. dorotocephala* to ethanol at concentrations between 0.01% and 1% and observed that the planarians exhibited concentration-related increases in C-shape movements (head of planarian was brought closer to tail laterally leading to a body shapes like the letter “C”). Therefore, the no-observed-adverse-effect level of ethanol in relation to freshwater planarians must be 0.01% or lower, as noted in one study (Ofoegbu et al., 2016).

The 24-h, 48-h, 72-h, and 96-h LC_{50} values of DMSO in relation to *D. japonica* were 4.03%, 3.67%, 3.56%, and 3.28%, respectively (Yuan et al., 2012). In addition, exposure to DMSO at concentrations higher than 0.1% and 0.5% caused alterations in planarians' activities of antioxidant enzymes and locomotion, respectively. Its interferences in motility, neoblast division, and gene expression patterns in *S. mediterranea* were also reported (Stevens et al., 2015). Thus, when DMSO is used as the solvent for test chemicals, its final concentration in the aqueous test solution should be no higher than 500 μL L^{-1} (0.05%) to eliminate its potential influence on the toxicity results (Stevens et al., 2015).

5.7. Environmental conditions during exposure

In addition to LC_{50} data obtained from previous tests, Supplementary Table S1 summarizes information regarding environmental conditions from those tests.

5.7.1. Temperature

The temperature during exposure to toxicants was maintained within the range of 18–26 °C for most studied species of planarians, including the aforementioned members of the genera *Dugesia*, *Girardia*, and *Schmidtea*. The “room temperature”, which is approximately 20–25 °C, appears to be suitable for conducting experiments with these freshwater planarians. This assertion was supported by Rivera and Perich (1994), who observed that planarians, including *G. dorotocephala* and *G. tigrina*, exhibited their highest survival rates when stored at 22 °C. However, 30 °C was harmful to these planarians (Rivera and Perich, 1994). By contrast, lower temperatures (in the range of 11–20 °C) was maintained for *P. felina* when used in an experiment; the effect of a higher temperature on this species remains unknown.

5.7.2. Water

Although natural and dechlorinated tap water have been used as water sources for storing freshwater planarians in laboratories and using them in experiments, reconstituted water is recommended because of some advantages, including: (1) ionic constitutions and their levels are known; (2) suspicions of potential interference and harmful substances in natural or tap water are eliminated; and (3) interlaboratory differences in experimental conditions are decreased, rendering further comparative study possible.

The pH values of rearing water and test solutions for planarians were maintained within the range of 6.8–7.8 (Supplementary Table S1). Maintaining planarians in more acidic ($pH < 6$) or basic ($pH > 8$) water reduces their survival rate and impairs asexual reproduction (Rivera and Perich, 1994).

Rivera and Perich (1994) observed that water hardness exerted no significant effect on the survival or asexual reproduction of studied planarian species, except for *G. tigrina*, whose survival rate exhibited a decreasing trend in soft water (< 48 mg L^{-1} $CaCO_3$). In addition, the researchers observed that freshwater planarians tended to tolerate low levels of salinity (NaCl; no more than 1 ppt) in water. Above 2 ppt, salinity began to exhibit lethality in some freshwater planarians.

In the literature, at least seven recipes have been applied for preparing reconstituted water for storing freshwater planarians during experiments (Supplementary Table S1). Generally, the ionic ingredients in reconstituted water are cations, including calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), and sodium (Na^{+}) ions, and anions, including bicarbonate (HCO_3^{-}), chloride (Cl^{-}), and sulfate (SO_4^{2-}) ions. The content of each ion in reconstituted water is summarized in Supplementary Fig. S1. Among all known recipes that have been applied for planarians, ionic levels in ASTM hard water (Ofoegbu et al., 2016), STM water (Villar et al., 1993), and ISO water (Wu et al., 2012a) fall within or close to the range of the 25th to 75th percentile (Supplementary Table S1 and Fig. S1), and perhaps could be proposed as universal recipes for reconstituted water in experiments using planarians. Softer reconstituted water with a lower overall ionic level has been used once each for *G. schubarti*, *G. tigrina*, and *P. tenuis* (Guecheva et al., 2003; Indeherberg et al., 1999; Knakievicz and Ferreira, 2008). However, such usage might be improper, at least for *G. tigrina*, because according to Rivera and Perich (1994), the survival rate of this species may decrease in soft water.

5.7.3. Photoperiod

Freshwater planarians are photophobic animals; this could be why some researchers have exposed planarians in completely dark environments (Franjevic et al., 2000; Hagstrom et al., 2015; Van Huizen et al., 2017). However, a previous study demonstrated that transferring planarians from a 12-h(L):12-h(D) cycle to a completely dark environment reduced the rhythm amplitude in their serotonin (Itoh and Igarashi, 2000). Therefore, to ensure regular physiological functions in test planarians, maintenance of a light–dark cycle during the experimental period should be considered. A 12-h(L):12-h(D) or 16-h(L):8-h (D) photoperiod cycle is commonly applied (Supplementary Table S1); however, whether divergence in the time of light–dark partitioning interferes with toxicity test results remains unknown.

5.8. Exposure period

Appropriate exposure schemes are critical in designed experiments to obtain useful results, particularly determining the optimal exposure period. As stated, freshwater planarians can be used to explore various types of potential toxicity due to chemicals. Notably, based on the studies addressed in Section 4, different exposure period are generally applied in planarian research to investigate various types of toxicities. Neurobehavioral changes, mobility, and mortality are often used as endpoints to evaluate the acute toxicity of chemicals in relation to planarians. Thus, in those studies, planarians have been exposed to a target chemical at a high (lethal or sublethal) concentration for a short period on a scale of minutes, hours, or days before endpoints were determined. When the effects of chemicals on biochemical parameters and the potential genotoxicity and teratogenicity of said pollutants have been investigated, exposure periods based on scales of days to weeks have often been applied. However, when focus is placed on the potential tumorigenicity or reproductive toxicity of chemicals, longer exposure periods based on scales of weeks to months appear to be necessary.

6. Conclusion

Invertebrate species can be used as alternatives to vertebrates in toxicology studies. Applying invertebrate species in eco- and environmental toxicology not only generally reduces the cost of maintenance and the difficulty of experimental manipulation but also yields useful toxicological information from individual to population or community level. Thus, some invertebrates have been proposed as models with standardized experimental protocols for screening chemicals. Freshwater planarians are distributed worldwide and can be easily obtained for scientific purposes. Thus far, results from many toxicological studies have collectively revealed that freshwater planarians are ideal test organisms for examining toxicity. Generally, the sensitivity of freshwater planarians is comparable to well-developed freshwater invertebrate models such as daphnids, as well as other aquatic animals. Freshwater planarians have been used to study genotoxicity, tumorigenicity, neurotoxicity, and reproductive toxicity; this suggests that they are potentially powerful tools in eco- and environmental toxicology for comprehensively examining the effects of chemicals and providing critical information generated from risk assessments. The necessity of standardizing the experimental protocol for using freshwater planarians has been asserted. Method standardization is crucial for conducting toxicological experiments with planarians in laboratories, particularly in comparative studies and routine screenings for toxic substances. Therefore, the optimized experimental method and conditions proposed in this paper can provide practical direction for researchers designing and conducting toxicological studies with planarians, regardless of the species used.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.05.057.

References

Alberdi, J., et al., 1996. Comparative acute toxicity of two herbicides, paraquat and glyphosate, to *Daphnia magna* and *D. spinulata*. Bull. Environ. Contam. Toxicol. 57, 229–235.
Alonso, A., Camargo, J., 2008. Ameliorating effect of chloride on nitrite toxicity to freshwater invertebrates with different physiology: a comparative study between amphipods and planarians. Arch. Environ. Contam. Toxicol. 54, 259–265.
Alonso, A., Camargo, J.A., 2006. Toxicity of nitrite to three species of freshwater invertebrates. Environ. Toxicol. 21, 90–94.
Alonso, A., Camargo, J.A., 2011. The freshwater planarian *Polycelis felina* as a sensitive species to assess the long-term toxicity of ammonia. Chemosphere 84, 533–537.
Alonso, A., Camargo, J.A., 2015. Ammonia toxicity to the freshwater planarian *Polycelis felina*: contrasting effects of continuous versus discontinuous exposures. Arch. Environ. Contam. Toxicol. 68, 689–695.
Behensky, C., et al., 2001. Quantitative analysis of turbellarian cell suspensions by fluorescent staining with acridine orange, and video microscopy. Belg. J. Zool. 131, 131–136.
Best, J.B., Morita, M., 1982. Planarians as a model system for in vitro teratogenesis studies. Teratog. Carcinog. Mutagen. 2, 277–291.
Best, J.B., Morita, M., 1991. Toxicology of planarians. Hydrobiologia 227, 375–383.
Bowden, H., et al., 1995. Assessment of the toxic and potential teratogenic effects of four glycol ethers and two derivatives using the hydra regeneration assay and rat whole embryo culture. Toxicol. Vitr. 9, 773–781.
Buttarelli, F.R., et al., 2008. Neuropharmacology and behavior in planarians: translations to mammals. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 147, 399–408.
Buttarelli, F.R., et al., 2000. Acetylcholine/dopamine interaction in planaria. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 125, 225–231.
Calevro, F., et al., 1998. Toxic effects of aluminium, chromium and cadmium in intact and regenerating freshwater planarians. Chemosphere 37, 651–659.
Calevro, F., et al., 1999. Bioassays for testing effects of Al, Cr and Cd using development in the amphibian *Pleurodeles waltl* and regeneration in the planarian *Dugesia etrusca*. Aquat. Ecosyst. Health Manag. 2, 281–288.
Calleja, M., et al., 1994. Human acute toxicity prediction of the first 50 MEIC chemicals by a battery of ecotoxicological tests and physicochemical properties. Food Chem. Toxicol. 32, 173–187.
Carolei, A., et al., 1975. Proposal of a new model with dopaminergic-cholinergic interactions for neuropharmacological investigations. Neuropsychobiology 1, 355–364.
Clay, R., 1996. Global agreement on alternative testing. Environ. Health Perspect. 104, 612.
Collins, T., 1987. Teratological research using in vitro systems. V. Nonmammalian model systems. Environ. Health Perspect. 72, 237.
Conners, D.E., Black, M.C., 2004. Evaluation of lethality and genotoxicity in the freshwater mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) exposed singly and in combination to chemicals used in lawn care. Arch. Environ. Contam. Toxicol. 46, 362–371.
Danny, J., Spehar, R., Mead, K., Shirin, C., 1991. Guidelines for Culturing the Japanese Medaka, *Oryszias latipes*. US Environmental Protection Agency, Washington, DC.
De Schamphelaere, K., et al., 2004. Reproductive toxicity of dietary zinc to *Daphnia magna*. Aquat. Toxicol. 70, 233–244.
Devaux, A., Larno, V., 1999. Genotoxicity assessment in the freshwater hydra using the comet assay. Comet Assay Workshop, Slovakia.
Ehlers, U., 1984. Phylogenetisches system der Plathelminthes. Verh. Nat. Ver. Hambg. 27, 291–294.
Famme, P., Knudsen, J., 1985. Anoxic survival, growth and reproduction by the freshwater annelid, *Tubifex* sp., demonstrated using a new simple anoxic chemostat. Comp. Biochem. Physiol. A Physiol. 81, 251–253.
Faria, M., et al., 2009. Multi-biomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to polychlorobiphenyls and metals. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 281–288.
Farrell, M.S., et al., 2008. Behavioral characterization of serotonergic activation in the flatworm Planaria. Behav. Pharmacol. 19, 177–182.
Foster, J., 1969. Malformations and lethal growths in planaria treated with carcinogens.

Natl. Cancer Inst. Monogr. 31, 683–691.
Foster, J.A., 1963. Induction of neoplasms in planarians with carcinogens. Cancer Res. 23, 300–303.
Franjevic, D., et al., 2000. The effects of zinc upon the survival and regeneration of planarian *Polycelis felina*. Biol. Bratisl. 55, 689–694.
Fukuhori, N., et al., 2005. Toxic effects of bisphenol A on sexual and asexual reproduction in *Hydra oligactis*. Arch. Environ. Contam. Toxicol. 48, 495–500.
Gélinas, M., et al., 2013. Temporal and seasonal variation in acetylcholinesterase activity and glutathione-S-transferase in amphipods collected in mats of *Lyngbya wollei* in the St-Lawrence River (Canada). Ecotoxicol. Environ. Saf. 94, 54–59.
Gómez-Oliván, L.M., et al., 2014. Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen in *Daphnia magna*. Drug Chem. Toxicol. 37, 391–399.
García-Medina, S., et al., 2013. Genotoxicity and oxidative stress induced by cadmium and zinc in the planarian, *Dugesia dorotocephala*. Afr. J. Biotechnol. 12, 4028–4038.
Gardner, G.R., et al., 1991. Carcinogenicity of Black Rock Harbor sediment to the eastern oyster and trophic transfer of Black Rock Harbor carcinogens from the blue mussel to the winter flounder. Environ. Health Perspect. 90, 53.
Gauthier, P.T., et al., 2016. Behavioural alterations from exposure to Cu, phenanthrene, and Cu-phenanthrene mixtures: linking behaviour to acute toxic mechanisms in the aquatic amphipod, *Hyalella azteca*. Aquat. Toxicol. 170, 377–383.
Gentile, L., et al., 2011. The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration. Dis. Models Mech. 4, 12–19.
Gerhardt, A., 2009. Screening the toxicity of Ni, Cd, Cu, ivermectin, and imidacloprid in a short-term automated behavioral toxicity test with *Tubifex tubifex* (Müller 1774) (Oligochaeta). Hum. Ecol. Risk Assess. 15, 27–40.
Goss, L.B., Sabourin, T.D., 1985. Utilization of alternative species for toxicity testing: an overview. J. Appl. Toxicol. 5, 193–219.
Grebe, E., Schaeffer, D.J., 1991a. Neurobehavioral toxicity of cadmium sulfate to the planarian *Dugesia dorotocephala*. Bull. Environ. Contam. Toxicol. 46, 727–730.
Grebe, E., Schaeffer, D.J., 1991b. Planarians in toxicology, standardization of a rapid neurobehavioral toxicity test using phenol in a crossover study. Bull. Environ. Contam. Toxicol. 46, 866–870.
Guecheva, T., et al., 2001. Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay). Mutat. Res. 497, 19–27.
Guecheva, T.N., et al., 2003. Stress protein response and catalase activity in freshwater planarian *Dugesia* (*Girardia*) *schubarti* exposed to copper. Ecotoxicol. Environ. Saf. 56, 351–357.
Hagstrom, D., et al., 2015. Freshwater planarians as an alternative animal model for neurotoxicology. Toxicol. Sci. 147, 270–285.
Hagstrom, D., et al., 2016a. Planarian brain regeneration as a model system for developmental neurotoxicology. Regeneration 3, 65–77.
Hagstrom, D., et al., 2016b. Planarian cholinesterase: in vitro characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity and reactivation. Arch. Toxicol. 1–11.
Hall, F., et al., 1986a. Neoplastic transformation in the planarian: I. Cocarcinogenesis and histopathology. J. Exp. Zool. A Ecol. Genet. Physiol. 240, 211–227.
Hall, F., et al., 1986b. Neoplastic transformation in the planarian: II. Ultrastructure of malignant reticuloma. J. Exp. Zool. A Ecol. Genet. Physiol. 240, 229–244.
Hase, S., et al., 2004. Characterization of novel genes expressed specifically in the sexual organs of the planarian *Dugesia ryukyuensis*. Int. J. Dev. Biol. 51, 345–349.
Heckmann, L., Connon, R., 2007. Culturing of *Daphnia magna*—standard operating procedure. Daphnia Res. Group Stand. Oper. Proced. 1, 1–8.
Hori, I., Kishida, Y., 1998. A fine structural study of regeneration after fission in the planarian *Dugesia japonica*. Hydrobiologia 383, 131–136.
Horvat, T., et al., 2005. Toxicity testing of herbicide norflurazon on an aquatic bioindicator species–the planarian *Polycelis felina* (Daly.). Aquat. Toxicol. 73, 342–352.
Hoshi, M., et al., 2003. Switch from asexual to sexual reproduction in the planarian *Dugesia ryukyuensis*. Integr. Comp. Biol. 43, 242–246.
Indeherberg, M.B., et al., 1999. Combining life-history and toxicokinetic parameters to interpret differences in sensitivity to cadmium between populations of *Polycelis tenuis* (Platyhelminthes). Ecotoxicol. Environ. Saf. 44, 1–11.
Itoh, M.T., Igarashi, J., 2000. Circadian rhythm of serotonin levels in planarians. Neuroreport 11, 473–476.
Jha, A.N., et al., 1996. Development of an in vivo genotoxicity assay using the marine worm *Platynereis dumerilii* (Polychaeta: Nereidae). Mutat. Res./Environ. Mutagen. Relat. Subj. 359, 141–150.
Kalafatić, M., et al., 2004. The impairments of neoblast division in regenerating planarian *Polycelis felina* (Daly.) caused by in vitro treatment with cadmium sulfate. Toxicol. Vitr. 18, 99–107.
Kapu, M.M., Schaeffer, D.J., 1991c. Planarians in toxicology. Responses of asexual *Dugesia dorotocephala* to selected metals. Bull. Environ. Contam. Toxicol. 47, 302–307.
Kato, C., et al., 2004. Motility recovery during the process of regeneration in freshwater planarians. Behav. Brain Res. 150, 9–14.
Kikuchi, M., et al., 2000. Screening of organophosphate insecticide pollution in water by using *Daphnia magna*. Ecotoxicol. Environ. Saf. 47, 239–245.
King, S.M., Patel-King, R.S., 2016. Planaria as a model system for the analysis of ciliary assembly and motility. In: Satir, P., Christensen, S. (Eds.), Cilia. Methods in Molecular Biology 1454 Humana Press, New York, NY.
Knakievicz, T., 2014. Planarians as invertebrate bioindicators in freshwater environmental quality: the biomarkers approach. Ecotoxicol. Environ. Contam. 9, 1–12.
Knakievicz, T., et al., 2008. Planarian neoblast micronucleus assay for evaluating genotoxicity. Chemosphere 72, 1267–1273.
Knakievicz, T., Ferreira, H.B., 2008. Evaluation of copper effects upon *Girardia tigrina* freshwater planarians based on a set of biomarkers. Chemosphere 71, 419–428.
Kobayashi, K., et al., 2002. Seasonal changes in the sexualization of the planarian *Dugesia ryukyuensis*. Zool. Sci. 19, 1267–1278.
Kobayashi, K., Hoshi, M., 2002. Switching from asexual to sexual reproduction in the planarian *Dugesia ryukyuensis*: change of the fissiparous capacity along with the sexualizing process. Zool. Sci. 19, 661–666.
Kovačević, G., et al., 2009. The effect of aluminium on the planarian *Polycelis felina* (Daly.). Water Air Soil Pollut. 196, 333–344.
Kustov, L., et al., 2014. Estimation of the toxicity of silver nanoparticles by using planarian flatworms. Altern. Lab. Anim. 42, 51–58.
Lacaze, E., et al., 2010. Genotoxicity assessment in the amphipod *Gammarus fossarum* by use of the alkaline comet assay. Mutat. Res./Genet. Toxicol. Environ. Mutagen. 700, 32–38.
Lagadic, L., Caquet, T., 1998. Invertebrates in testing of environmental chemicals: are they alternatives? Environ. Health Perspect. 106, 593.
Lau, A.H., et al., 2007. Freshwater planarians as novel organisms for genotoxicity testing: analysis of chromosome aberrations. Environ. Mol. Mutagen. 48, 475–482.
Lemieux, H., Warren, B.E., 2012. An animal model to study human muscular diseases involving mitochondrial oxidative phosphorylation. J. Bioenerg. Biomembr. 44, 503–512.
Li, M.-H., 2012a. Acute toxicity of benzophenone-type UV filters and paraben preservatives to freshwater planarian, *Dugesia japonica*. Toxicol. Environ. Chem. 94, 566–573.
Li, M.-H., 2013a. Acute toxicity of 30 pharmaceutically active compounds to freshwater planarians, *Dugesia japonica*. Toxicol. Environ. Chem. 95, 1157–1170.
Li, M.-H., 2013b. Acute toxicity of industrial endocrine-disrupting chemicals, natural and synthetic sex hormones to the freshwater planarian, *Dugesia japonica*. Toxicol. Environ. Chem. 95, 984–991.
Li, M.-H., 2014. Effects of bisphenol A, two synthetic and a natural estrogens on head regeneration of the freshwater planarians, *Dugesia japonica*. Toxicol. Environ. Chem. 96, 1174–1184.
Li, M.H., 2008. Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. Chemosphere 70, 1796–1803.
Li, M.H., 2012b. Survival, mobility, and membrane-bound enzyme activities of freshwater planarian, *Dugesia japonica*, exposed to synthetic and natural surfactants. Environ. Toxicol. Chem. 31, 843–850.
Li, M.H., 2016. Development of in vivo biotransformation enzyme assays for ecotoxicity screening: In vivo measurement of phases I and II enzyme activities in freshwater planarians. Ecotoxicol. Environ. Saf. 130, 19–28.
Lopes, K., et al., 2015. Method of isolation and characterization of *Girardia tigrina* stem cells. Biomed. Rep. 3, 163–166.
Lowe, J.R., et al., 2015. Ethanol exposure induces a delay in the reacquisition of function during head regeneration in *Schmidtea mediterranea*. Neurotoxicol. Teratol. 48, 28–32.
Mann, R.M., et al., 2010. A rapid amphipod reproduction test for sediment quality assessment: in situ bioassays do not replicate laboratory bioassays. Environ. Toxicol. Chem. 29, 2566–2574.
Matozzo, V., et al., 2005. Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. Mar. Pollut. Bull. 50, 1686–1693.
Medvedev, I., Komov, V., 2005. Regeneration of freshwater planarians *Dugesia tigrina* and Polycelis tenuis under the influence of methyl mercury compounds of natural origin. Rus. J. Dev. Biol. 36, 29–33.
Mitra, S., et al., 2003. Impact of CdCl2 on biochemical changes in planaria, *Dugesia bengalensis* Kawakatsu. Indian J. Exp. Biol. 41, 921–923.
Morimoto, R.I., et al., 1990. Stress Proteins in Biology and Medicine. Cold Spring Harbor Laboratory, Long Island, New York, USA.
Murray, M.R., 1927. The cultivation of planarian tissues in vitro. J. Exp. Zool. A Ecol. Genet. Physiol. 47, 467–505.
Neuhauser, E., et al., 1985. The toxicity of selected organic chemicals to the earthworm *Eisenia fetida*. J. Environ. Qual. 14, 383–388.
Neuhauser, E.F., et al., 1986. Comparative toxicity of ten organic chemicals to four earthworm species. Comp. Biochem. Physiol. C Comp. Pharmacol. 83, 197–200.
Nishimura, K., et al., 2007. Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. Dev. Neurobiol 67, 1059–1078.
Nishimura, K., et al., 2011. Regeneration of dopaminergic neurons after 6-hydroxydopamine-induced lesion in planarian brain. J. Neurochem. 119, 1217–1231.
Noreña, C., et al., 2015. Phylum Platyhelminthes. Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates. Academic Press, Cambridge, Massachusetts, USA, pp. 181–203.
Northcutt, R.G., 2012. Evolution of centralized nervous systems: two schools of evolutionary thought. Proc. Natl. Acad. Sci. USA 109, 10626–10633.
Ofoegbu, P.U., et al., 2016. Toxicity of tributyltin (TBT) to the freshwater planarian *Schmidtea mediterranea*. Chemosphere 148, 61–67.
Oliveira, L.L., et al., 2015. Evaluation of ecotoxicological effects of drugs on *Daphnia magna* using different enzymatic biomarkers. Ecotoxicol. Environ. Saf. 119, 123–131.
Onwumere, E.A., Wells, M.R., 2015. Metabolism of p,p'-DDT by the freshwater planarian *Phagocata gracilis*. Bull. Environ. Contam. Toxicol. 31, 18–21.
Oviedo, N.J., et al., 2008a. Planarians: a versatile and powerful model system for molecular studies of regeneration, adult stem cell regulation, aging, and behavior. Cold Spring Harb. Protoc. 2008 (pdb. emo101).
Oviedo, N.J., et al., 2008b. Establishing and maintaining a colony of planarians. Cold Spring Harb. Protoc. 2008 (pdb. prot5053).
Pagán, O.R., 2017. Planaria: an animal model that integrates development, regeneration and pharmacology. Int. J. Dev. Biol. 61, 519–529.
Pagán, O.R., et al., 2006. Toxicity and behavioral effects of dimethylsulfoxide in planaria. Neurosci. Lett. 407, 274–278.
Pagan, O.R., et al., 2009. The flatworm planaria as a toxicology and behavioral

pharmacology animal model in undergraduate research experiences. J. Undergrad. Neurosci. Educ. 7, A48–A52.
Pagan, O.R., et al., 2015. Evidence of nicotine-induced, curare-insensitive, behavior in planarians. Neurochem. Res. 40, 2087–2090.
Palladini, G., et al., 1996. A pharmacological study of cocaine activity in planaria. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 115, 41–45.
Papenfus, K.F., 1983. Effects of Methylmercury on Regeneration of Brain and Behavior in Decapitated Planarians. Master of Science thesis. Colorado State University, Fort Collins, Colorado, USA.
Phillips, J., et al., 1974. Metabolism of DDT by the freshwater planarian, *Phagocata velata*. Bull. Environ. Contam. Toxicol. 12, 355–358.
Piontek, M., 1999. Use of a planarian *Dugesia tigrina* Girard in the studies of acute toxicity of organic substances. Pol. Arch. Hydrobiol. 46.
Plusquin, M., et al., 2012. Physiological and molecular characterisation of cadmium stress in *Schmidtea mediterranea*. Int. J. Dev. Biol. 56, 183–191.
Prokai, D., et al., 2013. An exploratory evaluation of tyrosine hydroxylase inhibition in planaria as a model for parkinsonism. Int. J. Mol. Sci. 14, 23289–23296.
Quinn, B., et al., 2009. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, *Hydra attenuata*. Sci. Total Environ. 407, 1072–1079.
Raffa, R.B., et al., 2001. Quantitative assessment of dopamine D2 antagonist activity using invertebrate (Planaria) locomotion as a functional endpoint. J. Pharmacol. Toxicol. Method. 45, 223–226.
Raffa, R.B., Rawls, S.M., 2009. Planaria: a model for drug action and abuse. Landes Bioscience.
Rawls, S.M., et al., 2011. Nicotine behavioral pharmacology: clues from planarians. Drug Alcohol Depend. 118, 274–279.
Reddien, P.W., Alvarado, A.S., 2004. Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. 20, 725–757.
Reed, B., Jennings, M., 2011. Guidance on the housing and care of zebrafish. South.: R. Soc. Prev. Cruel. Anim.
Reutgard, M., et al., 2014. Embryo development of the benthic amphipod *Monoporeia affinis* as a tool for monitoring and assessment of biological effects of contaminants in the field: a meta-analysis. Ecol. Indic. 36, 483–490.
Rink, J.C., 2013. Stem cell systems and regeneration in planaria. Dev. Genes Evolut. 223, 67–84.
Rivera, V., Perich, M., 1994. Effects of water quality on survival and reproduction of four species of planaria (Turbellaria: Tricladida). Invertebr. Reprod. Dev. 25, 1–7.
Robb, S.M., et al., 2015. SmedGD 2.0: the *Schmidtea mediterranea* genome database. Genesis 53 (535-46).
Rodrigues, A.C., et al., 2016. Behavioural responses of freshwater planarians after short-term exposure to the insecticide chlorantraniliprole. Aquat. Toxicol. 170, 371–376.
Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. 22, 81–113.
Rogge, R.W., Drewes, C.D., 1993. Assessing sublethal neurotoxicity effects in the freshwater oligochaete, *Lumbriculus variegatus*. Aquat. Toxicol. 26, 73–89.
Russell, W., Burch, R., 1959. The Principle of Humane Experimental Technique. Methuen, London, England.
Sabourin, T., et al., 1985. The efficacy of three non-mammalian test systems in the identification of chemical teratogens. J. Appl. Toxicol. 5, 227–233.
Saló, M., Agata, K., 2012. The Planaria Model System. Int. J. Dev. Biol. 56, 1–196.
Sarnat, H.B., Netsky, M.G., 2002. When does a ganglion become a brain? Evolutionary origin of the central nervous system. Semin. Pediatr. Neurol. 9, 240–253.
Schürmann, W., Peter, R., 2001. Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts. Belg. J. Zool. 131, 123–130.
Schaeffer, D.J., 1993. Planarians as a model system for in vivo tumorigenesis studies. Ecotoxicol. Environ. Saf. 25, 1–18.
Schockaert, E.R., et al., 2008. Global diversity of free living flatworms (Platyhelminthes, "Turbellaria") in freshwater. In: Balian, E.V. (Ed.), Freshwater Animal Diversity Assessment. Springer, Netherlands, Dordrecht, pp. 41–48.
Sengel, C., 1960. In vitro culture of regeneration blastemata in planaria. J. Embryol. Exp. Morphol. 8, 468–476.
Servizi, J., et al., 1987. Acute toxicity of Garlon 4 and Roundup herbicides to salmon, Daphnia, and trout. Bull. Environ. Contam. Toxicol. 39, 15–22.
Sheiman, I.M., Kreshchenko, I.D., 2015. Regeneration of planarians: experimental object. Ontogenez 46, 3–12.
Simanov, D., et al., 2012. The flatworm macrostomum lignano is a powerful model organism for ion channel and stem cell research. Stem Cells Int. 2012, 167265.
Stevens, A.S., et al., 2015. Toxicity profiles and solvent–toxicant interference in the planarian *Schmidtea mediterranea* after dimethylsulfoxide (DMSO) exposure. J. Appl. Toxicol. 35, 319–326.
Stevens, A.S., et al., 2017. Stem cell proliferation patterns as an alternative for in vivo prediction and discrimination of carcinogenic compounds. Sci. Rep. 7, 45616.
Tallarida, C.S., et al., 2014. Ethanol and cocaine: environmental place conditioning, stereotypy, and synergism in planarians. Alcohol 48, 579–586.
Tehseen, W., et al., 1992. A scientific basis for proposed quality assurance of a new screening method for tumor-like growths in the planarian, *Dugesia dorotocephala*. Qual. Assur. 1, 217–229.
Thain, J., 1986. Toxicity of TBT to bivalves: effects on reproduction, growth and survival. IEEE 1306–1313 (OCEANS'86).
Ton, S.-S., et al., 2012. Evaluation of acute toxicity and teratogenic effects of disinfectants by *Daphnia magna* embryo assay. Environ. Pollut. 168, 54–61.
Van Huizen, A.V., et al., 2017. Methylisothiazolinone toxicity and inhibition of wound healing and regeneration in planaria. Aquat. Toxicol. 191, 226–235.
Ventura-Lima, J., et al., 2007. Toxicological responses in *Laeonereis acuta* (Annelida, Polychaeta) after arsenic exposure. Environ. Int. 33, 559–564.
Venturini, G., et al., 1989. A pharmacological study of dopaminergic receptors in planaria. Neuropharmacology 28, 1377–1382.
Villar, D., et al., 1993. Toxicity of organophosphorus pesticides to *Dugesia dorotocephala*. Bull. Environ. Contam. Toxicol. 51, 80–87.
Voura, E.B., et al., 2017. Planarians as models of cadmium-induced neoplasia provide measurable benchmarks for mechanistic studies. Ecotoxicol. Environ. Saf. 142, 544–554.
Vries, E.J., Sluys, R., 1991. Phylogenetic relationships of the genus *Dugesia* (Platyhelminthes, Tricladida, Paludicola). J. Zool. 223, 103–116.
Vu, H.T., Keough, M.J., Long, S.M., Pettigrove, V.J., 2017. Effects of two commonly used fungicides on the amphipod *Austrochiltonia subtenuis*. Environ. Toxicol. Chem. 36, 720–726.
Welch, W.J., 1993. How cells respond to stress. Sci. Am. 268, 56–64.
Wu, J.-P., et al., 2011. The preferential accumulation of cadmium in the head portion of the freshwater planarian, *Dugesia japonica* (Platyhelminthes: Turbellaria). Metallomics 3, 1368–1375.
Wu, J.-P., et al., 2012a. Bioaccumulation and toxicodynamics of cadmium to freshwater planarian and the protective effect of N-acetylcysteine. Arch. Environ. Contam. Toxicol. 63, 220–229.
Wu, J.-P., et al., 2014. Cadmium neurotoxicity to a freshwater planarian. Arch. Environ. Contam. Toxicol. 67, 639–650.
Wu, J.-P., Li, M.-H., 2015. Inhibitory effects of pain relief drugs on neurological enzymes: implications on their potential neurotoxicity to aquatic animals. Environ. Toxicol. Pharmacol. 39, 898–905.
Wu, J.-P., Li, M.-H., 2017. Low uptakes of Cd, Cu, and Zn in *Dugesia japonica*, a freshwater planarian with higher tolerance to metals. Chem. Ecol. 33, 257–269.
Wu, J.-P., et al., 2015. Disturbances to neurotransmitter levels and their metabolic enzyme activity in a freshwater planarian exposed to cadmium. Neurotoxicology 47, 72–81.
Wu, J.-P., et al., 2012b. In vivo and in vitro metabolism of tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), by the freshwater planarian, *Dugesia japonica*. Chemosphere 87, 1341–1347.
Yuan, Z., et al., 2012. Effects of dimethylsulfoxide on behavior and antioxidant enzymes response of planarian *Dugesia japonica*. Toxicol. Ind. Health 28, 449–457.
Zeeshan, M., et al., 2016. ROS dependent copper toxicity in Hydra-biochemical and molecular study. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 185, 1–12.
Zhang, H.-C., et al., 2017. Genotoxicity evaluation of an urban river on freshwater planarian by RAPD assay. Bull. Environ. Contam. Toxicol. 98, 484–488.
Zhang, X., et al., 2016. Induction ofhsp70, hsp90, and catalase activity in planarian D*ugesia japonica* exposed to cadmium. Toxicol. Ind. Health 32, 1373–1380.
Zhang, X., et al., 2013. Effects of chlorpyrifos on acute toxicity, mobility and regeneration of planarian *Dugesia japonica*. Fresenius Environ. Bull. 22, 2610–2615.
Zhang, X., et al., 2014. Mortality and antioxidant responses in the planarian (*Dugesia japonica*) after exposure to copper. Toxicol. Ind. Health 30, 123–131.